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Development of suspension polymerized molecularly imprinted beads with metergoline as template and application in a solid phase extraction procedure towards ergot alkaloids.

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Abstract

The first successfully developed molecularly imprinted polymer towards six ergot alkaloids and their respective epimers is described. A new imprinting molecule, metergoline, was used as template analogue in the production of suspension polymerized beads. These spherical particles functioned as selective sorbent in a solid-phase extraction column. The application of this column in the clean-up of barley samples prior to liquid chromatography coupled with tandem mass spectrometry allowed a simple and cost-efficient sample preparation. The performance of the imprinted polymer and a non-imprinted control polymer was evaluated. This includes the determination of the recovery values and the matrix effect of each of the 12 tested ergot alkaloids as well as a cross-reactivity study with 25 common mycotoxins. The binding isotherms were obtained for metergoline, thus allowing comparison with other (imprinted) sorbents. A comparison between bulk and suspension polymerization is provided to determine the appropriate production technique.

Introduction

Ergot alkaloids are mycotoxins produced by *Claviceps* species, specifically *C. purpurea*. These fungi can be found on cereals and produce sclerotia, which vary in composition and concentration of ergot alkaloids. The sclerotia are harvested together with the grains and only up to 80% of the kernels can be removed with the mechanical separation techniques currently available. Especially in periods of drought these techniques become unreliable as smaller sclerotia, similar in size as the grains, are produced. Generally, sclerotia are also broken during transport allowing them to enter the food chain more easily.

These ergot alkaloids possess toxicity due to interactions with α -adrenergic, serotonergic and dopaminergic receptors^{1,2,3}. An excess intake by humans can cause nausea, convulsions, hallucinations, vasoconstriction and even lead to gangrenous symptoms and abortion. Animals are infected through consumption of contaminated feed. This is a widespread phenomenon in livestock leading to adverse effects ranging from weight loss to death of infected animals^{4,5,6,2,7}.

The European Food Safety Authority (EFSA) stated in 2005 that the degree of variability in ergot alkaloid pattern in relation to fungal species, host plant and geographical distribution is not known⁵. Six ergot alkaloids, ergometrine, ergosine, ergotamine, ergocristine, ergocryptine and ergocornine, which are considered the most important ones according to the EFSA, are depicted in Figure 1. An isomerization from the biological active R-form to the biological inactive S-form occurs at the C₈ stereogenic center of the tetracyclic ergoline-structure in a reversible epimerization reaction^{8,9,6}. The suffix *-ine* is used in the nomenclature if the side chain is present in the R-form at the C₈ stereogenic center, for example ergotamine. The side chain is present in the S-orientation in the correspondent epimer which is expressed by the suffix *-inine*, for example ergotaminine. This reaction is promoted in humid, aqueous and acidic environment which corresponds to the storage conditions of cereals in silage⁴. Because of the reversibility of this process, it is important to include the S-epimers in the analytical methods as well. Omitting them might lead to an underestimation of the total biological active ergot alkaloid content.

An in-house high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) method selective toward the 6 ergot alkaloids and their respective epimers was developed by Diana Di Mavungu et al¹⁰. However, an important feature is the clean-up of samples prior to analysis. The presence of matrix interferences leads to ion suppression or enhancement in many cases^{11,12}. In this investigation a molecularly imprinted polymer

(MIP)^{13,14,15,16} was developed and applied as a sorbent in a molecularly imprinted solid phase extraction (MISPE)^{17,18} protocol. This approach was chosen as an alternative clean-up procedure in replacement of the commonly used extensive procedure involving liquid-liquid extractions, centrifuging, filtration steps and solid phase extractions. Literature has shown the validity of imprinted polymers, possessing a specificity and recovery similar to the results obtained by ion-exchange or immunoaffinity columns^{19,20}. Contrary to natural antibodies, MIPs have no animal origin, are robust, thermally stable and can be reused multiple times. Once the parameters of the production process are optimized, the MIPs are produced in a short period.

To our knowledge, MIPs have not yet been successfully developed for the intended single ergot alkaloid compounds, nor has a group-selective MIP. A successful imprinted polymer towards lysergic acid diethylamine (LSD) was developed by Chapuis-Hugon et al.²¹ using ergometrine as template molecule. However, the selectivity towards the six ergot alkaloids and epimers was not tested. Also, template bleeding of the ergometrine may influence accurate determination of this compound. An imprinted polymer containing dopamine/serotonin-like binding sites was produced by Suedee et al.²². The recoveries were determined for several ergot alkaloids, but the values were situated between 30 – 55%.

This paper elaborates on the first successfully developed MIP towards the six ergot alkaloids and their respective epimers as displayed in Figure 1. A new imprinting molecule, metergoline, was used as template. This cheap and harmless compound could also avoid problems associated with template bleeding by the consequent analysis with LC-MS/MS. Its structural resemblance with the ergot alkaloids enabled the production of a group-selective MIP. This is a great advantage and has not been reported before. Its application as a sorbent in an SPE column was validated in the clean-up of barley samples.

Figure 1. Up: overview of the six ergot alkaloids. All of these compounds share the same common basic structure (left) but differ in side chains (right). The wavy line in the side chains' structure indicates their connection to the basic structure via a N-C binding at the amide bond. The side chains are depicted in the R-configuration at the C₈ position whereas their epimers are in the S-configuration. The side chains are named from left-to-right, first row: ergocryptine, ergotamine, ergometrine; second row: ergocristine, ergosine and ergocornine. Down: the template molecule, metergoline.

Experimental section

Materials

Polyvinyl alcohol (PVA), methacrylic acid (MAA), ethylene glycol dimethyl acrylate (EGDMA), triethylamine (TEA), NH₄HCO₃, methylergometrine (MEM) and dihydroergotamine (DHET) were bought from Sigma-Aldrich (Bornem, Belgium). 2,2-azobisisobutyronitrile (AIBN), acetone, ethyl acetate and chloroform were acquired from Acros Organics (Geel, Belgium). Standards of metergoline, ergotamine (Et), ergotaminine (Etn), ergocornine (Eco), ergocorninine (Econ), ergocryptine (Ekr), ergocryptinine (Ekrn), ergocristine (Ecr), ergocristinine (Ecrn), ergosine (Es), ergosinine (Esn), ergometrine (Em) and ergometrinine (Emn) were purchased from Coring System Diagnostix (Gernsheim, Germany). Ultra purified water was obtained using a Milli-Q Gradient system (Millipore, Brussels, Belgium). Na₂HPO₄, Methanol Hipersolv, hexane and acetonitrile (Analar Normapur) were bought from VWR International (Leuven, Belgium). KH₂PO₄ was supplied by Merck (Darmstadt, Germany) and LC-MS grade MeOH was obtained from Biosolve (Valkenswaard, the Netherlands).

Empty 3 ml SPE cartridges and the corresponding frits with pore size 20 µm were purchased from Agilent (Diegem, Belgium).

The mycotoxin standards used in the cross-reactivity study were the following. Nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), ochratoxin A (OTA), zearalenone (ZEN), aflatoxin B1 (AF-B1), aflatoxin B2 (AF-B2), neosolaniol (NEO), fusarenon-X (F-X), aflatoxin G1 (AF-G1), aflatoxin G2 (AF-G2), citrinin (CIT), HT-2 toxin (HT-2), alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), fumonisin B1 (FB-1), fumonisin B2 (FB-2), sterigmatocystin (STERIG) and beauvericin (BEAU) were all from Sigma-Aldrich (Bornem, Belgium). Diacetoxyscirpenol (DAS) and T-2 toxin (T2) were obtained from Biopure (Tulln, Austria). Fumonisin B3 (FB-3) was purchased from Medical Research Council South Africa (Tygerberg, South Africa) and roquefortine C (Roq.C) was obtained from Enzo Life Science (Lorrach, Germany).

Methods

Suspension polymerization. Two stock solutions were prepared prior to polymerization: the dispersive phase and the continuous phase. The former was composed of 0.278 g AIBN dissolved in 10 ml of the porogen, chloroform. The latter comprised 0.101 g PVA, which acts as an emulsion stabilizer, dissolved in 20 ml water. The template (metergoline), functional monomer (MAA) and cross-linker (EGDMA) were added in a 1:6:24 molar ratio²³. The polymerization mixture was prepared by transferring 57.8 mg of metergoline to a 25 ml round-bottom flask together with 0.90 ml of the dispersive phase stock solution, 73 µl MAA, 0.65 ml EGDMA and 4.7 ml of the continuous phase stock solution. The content was mixed during 30 seconds at 17000 rpm using the Turrax mixer (IKA Werke, Staufen, Germany). The flask was flushed with nitrogen, sealed off and placed between UV lights ($\lambda = 365$ nm; 150 mW/cm²) after which the reaction was initiated. After 2 hours of irradiation at room temperature, the reaction was stopped by removing the flask from the UV lights. The beads were collected in an empty SPE cartridge provided with a frit and dried in an oven at 60°C. The template was subsequently removed by rinsing the MIPs with an MeOH/TEA (95/5) mixture for 20 hours in a Soxhlet extractor. Non-imprinted polymers (NIPs) were produced similar as the MIPs; however, no template was included in their production. Fifty mg of imprinted polymer was packed in an empty SPE cartridge between two frits, thus rendering an MISPE-column. An NISPE-column was obtained alike with 50 mg of non-imprinted polymer.

Bulk polymerization. The same amounts of metergoline, MAA, EGDMA and dispersive phase as used in the preparation of the beads obtained by suspension polymerization, were utilized. However, the continuous phase was omitted. These compounds were transferred to a test tube and homogenized at 17000 rpm during 30 seconds using the Turrax mixer. After flushing with nitrogen, the test tube was sealed off and put between the UV lights ($\lambda = 365$ nm; 150 mW/cm²). The mixtures were subjected to different reaction times: 2h, 3h, 4h and 20h respectively. A corresponding NIP was prepared for each of the imprinted polymers. A monolith was obtained after reaction which was subsequently crushed and ground. The polymers were rinsed by soxhlet extraction similar as mentioned above.

Extraction procedure of barley samples. Five g of ground barley were weighed in a tube and 40 ml of extraction solvent - ethyl acetate/MeOH/NH₄HCO₃ buffer (buffer 16 g/l, pH = 8.5), 70/20/10, v/v/v - were added. The tube was tumbled for 30 min using an end-over-end tumbler (Exacta, Deurne, Belgium) after which it was centrifuged at 3000g during 15 min. The supernatant was fractionized in quantities of 10 ml which were evaporated under a nitrogen stream. These dry extracts were used as described further in this section.

Molecular imprinted solid-phase extraction (MISPE) procedure. The cartridge was conditioned with 2 ml of ACN. The dry sample obtained after extraction was dissolved in 250 µl of loading solvent, loaded onto the columns and allowed to interact for five minutes. The loading solvent was composed of KH₂PO₄/Na₂HPO₄ buffer at pH=7 and acetonitrile in a 20/80 ratio. A washing step was performed with 2 ml of distilled water after which the analytes were eluted with 3 ml of MeOH/TEA (95/5). This elution was performed at a rate of one drop of eluate per three seconds, which corresponds to a flow rate of 0.6 ml/min. The collected eluate was evaporated to dryness with nitrogen and dissolved in injection solvent, MeOH/ACN/H₂O (20/40/40), for LC-MS/MS analysis.

LC-MS/MS analysis. The HPLC equipment (Alliance 2695) and mass spectrometer (Quattro LCZ Micromass) were purchased from Waters (Milford, MA, USA). The column used for HPLC separation was an X-Bridge, C18, 3.5 µm, 2.1×150 mm (Waters, Zellik, Belgium). A gradient of two mobile phases (MF), MFA (H₂O/NH₄HCO₃-buffer/MeOH, 85/5/10) and MFB (H₂O/NH₄HCO₃-buffer/MeOH, 5/5/90) was applied for the separation of the ergot alkaloids as described by Diana Di Mavungu et al¹⁰.

Determination of recoveries, matrix effect and limits of detection/quantification. Barley samples were spiked at 4 µg/kg with a mixture of the six ergot alkaloids and their epimers before loading onto the column and compared to samples which were spiked after elution. The difference between these peak areas obtained by LC-MS/MS was used to determine the recovery. These values indicate the performance of the sorbents as such. Spiking during the loading step was performed by replacing 100 µl of ACN of the loading solvent with 100 µl of an 0.2 µg/ml solution of ergot alkaloid standards dissolved in ACN. Spiking after elution was done by adding 100 µl of the standard solution to the eluate prior to evaporation of the solvent.

Additionally, tests were conducted which are representative for the performance of the entire analysis procedure. The raw barley was spiked with 100 µl of 0.2 µg/ml ergot alkaloid mixture after milling in order to simulate the analysis of contaminated samples. The entire analysis procedure was performed including the extraction, centrifugation, re-dissolution in loading solvent, MIPSE-/NISPE- procedure and re-dissolving for LC-MS/MS analysis. The recovery was compared to uncontaminated barley samples spiked after elution.

The R- and S-isomers are connected by the epimerization reaction. Therefore, recovery values will be given for the sum of both epimers for each ergot alkaloid as the single values are prone to fluctuations from one sample or measurement to the other. The summation of for example ergotamine (Et) and ergotaminine (Etn) will be indicated as Et(n) in the nomenclature in this article. The other ergot alkaloids and their epimers will be denoted similarly.

Simultaneously, a comparison of the peak areas acquired with LC-MS/MS between a sample spiked after elution and pure standard solution was used to determine the matrix effect^{11,24}. The matrix effect was also evaluated in a second way using post-column injection^{25,12,26}. A syringe pump was connected to the HPLC column effluent using a tee. Standard solutions of single ergot alkaloid compounds were introduced at a constant rate and thus rendered a constant electrospray ionization response. By performing a gradient run without sample and one with a blank sample after the MISPE procedure, the influence of matrix compounds could be evaluated.

Two internal standards, dihydroergotamine and methylergometrine, were included at 5 µg/kg in the spiking mixtures used in all the experiments described in this section to correct for fluctuations due to the MS/MS equipment. The limit of detection (LOD) was defined as the signal-to-noise ratio of 3 or higher obtained by LC-MS/MS analysis. Accordingly, the quantification limit was determined as a ratio equal to 10 or higher.

Equilibrium experiments. Two series of imprinted and non-imprinted beads each, 5 mg and 50 mg respectively, were transferred in Eppendorf tubes. Subsequently, 1 ml of loading solvent, spiked with varying concentrations of metergoline in the range of 0.005 to 0.150 µmol/ml, was added. Eppendorf tubes without polymer were also included to determine the response of the initial spike solution. The tubes were shaken overnight (16h) on an end-over-end tumbler prior to centrifugation (15 min at 14000 g). The supernatant was collected, evaporated under nitrogen and re-dissolved in 500 µl of mobile phase prior to LC-MS/MS analysis. The bound amount of metergoline was determined as the difference in concentration between the initial spike solution and the concentration of the supernatant after equilibration (= free concentration).

Scatchard analysis. The binding isotherms obtained through the equilibrium experiments can be transformed to linear equations using the scatchard equation (eq.1).

$$\frac{B}{[F]} = \frac{B_{max} - B}{K_D} \quad (\text{eq.1})$$

where B is the amount of metergoline bound to the polymer at equilibrium, [F] is the concentration of free metergoline in solution (not to be mistaken with the initial concentration in solution), B_{max} is the apparent maximum number of binding sites and K_D is the apparent dissociation constant.

Cross-reactivity. The selectivity of the MIP and NIP sorbents was evaluated through determination of the recoveries of non-analogue structures. A cross-reactivity study was performed with 25 frequently occurring mycotoxins which represent a broad range of molecular structures. These compounds are mentioned in the materials section. Triplicate experiments were conducted where barley samples were spiked prior to loading onto the column and compared to samples spiked after elution. The LC-MS/MS method and the preparation of the spike solutions were performed according to the method developed by Monbaliu et al.^{27,28}.

Results and discussion

Suspension polymerization was applied to avoid the disadvantages which arrive from bulk polymerized MIPs^{16,29,18}. The crushing of the bulk-monolith was a time-consuming and wasteful process which resulted in

particles with an irregular size and shape. This irregularity led to a poor packing of the particles in an SPE-column and as a consequence, the efficiency was lower (data not shown). In contrast, the beads were directly prepared and rinsed with little amount of polymer wasted using suspension polymerization. A main advantage of this technique was the ability to control the size range in connection with a relatively narrow size distribution of the particles. This rendered a good packing of the beads with chromatographic properties such as a good flow rate and a low back-pressure. Large particles ($>100\ \mu\text{m}$) will allow a rapid flow of sample volume, whereas small particles ($<1\ \mu\text{m}$) possess a higher surface/volume ratio. An optimum between a low back pressure and sufficient surface for interaction is obtained for particles with a diameter of $25 - 75\ \mu\text{m}$, as in commercially available columns.

The size distribution (Figure 2) of the spherical particles was measured using a laser diffraction system, the Mastersizer S (Malvern Instruments, Worcestershire, UK). Eighty percent of the imprinted particles were situated in the $12\ \mu\text{m} - 58\ \mu\text{m}$ range with an average diameter of $30\ \mu\text{m}$. The NIPs possessed a slightly larger diameter, 80% of the beads were found in the range between $21\ \mu\text{m}$ and $95\ \mu\text{m}$, with an average of $45\ \mu\text{m}$. Additionally, scanning electron microscopy (FEI Phenom desktop SEM, Benelux Scientific, Nazareth, Belgium) was used to visualize the polymers. The images showed uniformly shaped, spherical beads with a smooth surface. Some particles were ruptured or broken due to mechanical friction thus revealing the internal structure: a breadcrumb-like structure. Diameter measurements performed on the SEM images confirmed the results obtained by the laser diffraction system. The polymers' dimensions were situated in the suitable range for the application as SPE sorbent.

Figure 2. From left to right: SEM picture and size distribution of MIP, SEM photograph and size distribution of NIP and finally a SEM image of the internal structure of the imprinted polymer. The size distribution graphs represent the volume percentage (in %) in function of the particle size (in μm).

The recoveries of the ergot alkaloids for the polymers manufactured by both polymerization techniques were compared to establish the most suitable production technique. The reaction times of the bulk-produced MIPs and NIPs were varied to investigate the effect towards performance. The lowest recoveries were obtained for the non-imprinted polymer (NIP3, 19% - 23%) and the highest values belonged to the imprinted polymers (MIP1 & MIP2, 56% - 71%), all of which were produced through suspension polymerization. The recoveries of all the bulk particle batches, both imprinted and non-imprinted, were situated between these former NIP and MIPs. The imprinted particles displayed lower recoveries and less difference with their respective NIPs in all cases. The good recoveries for MIP1 and MIP2 as well as the contrast with the values for NIP3, indicate the better performance of the suspension polymerized beads in comparison to the bulk-polymerized particles when using the MISPE procedure as described in the methods section.

Loading step. An MIP does not possess an intrinsic selectivity, an equally important aspect lies in the conditions in which the MIP is applied afterwards. Effective rebinding of the analyte will only occur if the production conditions of the polymer, like swelling, can be imitated during the loading step³⁰. It is important to maximize the interactions between the analytes and the MIP during the loading step to secure a high recovery. Adjusting the pH values of the loading solvent often helps to decrease the breakthrough of the desired compounds during the loading step¹⁴. The influence of pH in the range of pH 2 – 9 was investigated using phosphate buffers and an optimum was reached when using a buffer at pH = 7 (data not shown). In this conditions, the carboxylic acid present on MAA ($\text{pK}_a = 4,58 \pm 0,11$) will be deprotonated while the tertiary amines of the ergot alkaloids ($\text{pK}_a \sim 9,60 \pm 0,60$ except Em(n) with $\text{pK}_a = 14,16 \pm 0,10$) will be protonated. This facilitates the formation of ionic interactions between the alkaloids and the functional monomers of the polymer and results in an increased retention. These tests have led to the optimized loading step, described in the experimental section.

Performance of the MIP and NIP

Recovery. This is an important criterion because it gives an indication of the binding efficiency of the sorbents and it is necessary as correction factor when analyzing unknown samples. The values of the MIP recoveries were situated between 65% - 79% and those of the NIPs varied between 22% - 33% for all compounds as displayed in Table 1. This clearly demonstrates the difference in recovery between the imprinted and non-imprinted sorbents in the applied conditions. The raised values of the ergot alkaloids towards the MIPs in comparison with the NIPs indicate an elevated affinity which is typical for the presence of high(er) selective binding sites on the former. According to Annex II of the Commission Regulation (EC) 401/2006, acceptable recovery values are situated between 60% - 120%. The MIP performance was well within this interval.

As described in the methods section, a similar experiment was conducted with inclusion of the extraction procedure's influence on the recovery. Comparable recovery data as in the previous paragraph was obtained for all the ergot alkaloids except for ergometrin(in)e where a decrease of 10%-15% was noticed. This may be explained by a loss during the extraction step due to the lower hydrophobic nature of Em(n) and the extraction with high amounts of ethyl acetate. Again, a distinct difference in recovery values between MIP and NIP can be seen in Table1, indicating the overall good performance of the procedure.

	Em(n)	Es(n)	Et(n)	Eco(n)	Ekr(n)	Ecr(n)
MIP	79 ± 9	71 ± 7	71 ± 6	65 ± 7	68 ± 14	73 ± 15
NIP	33 ± 7	22 ± 7	23 ± 3	24 ± 2	25 ± 5	22 ± 4
MIP	56 ± 2	66 ± 7	71 ± 9	65 ± 3	69 ± 13	68 ± 7
NIP	23 ± 4	19 ± 2	19 ± 3	20 ± 3	21 ± 5	19 ± 1

Table 1. Summary of the recovery values (in %) and standard deviations (in %) for all ergot alkaloids towards the MIP and NIP. Up: spiking of the samples was performed after extraction of the barley samples. Down: spiking was done prior to extraction of the samples. The experiments were performed in triplicate on different days and different samples which were spiked at 4 µg/kg.

Limits of detection (LOD) and quantification (LOQ). Ergometrine (Em) and ergometrinine (Emn) both possessed an LOQ of 10 µg/kg. All other compounds could be quantified at 1 µg/kg except for ergosine (Es) and ergocristinine (Ecrn) which had LOQ's of respectively 2 and 3 µg/kg. Besides Em, Emn, Es and Ecrn the S/N ratios of the other compounds indicated that LOD values below 1 µg/kg could be established. However, lower limits were not further investigated because these low concentrations do not pose a threat towards human or animal health⁵.

Equilibrium experiments

Binding isotherms The MIP efficacy has been evaluated in the section above by means of recovery determination. This is a good indicator which, however, is only valid for the specific conditions in which it is applied including the concentrations and volumes used in the MISPE procedure. A more general tool for the evaluation of MIP's performance is the binding isotherm^{31,32}. To our knowledge no MIP towards metergoline has been produced up to now, but inclusion of the binding isotherms will allow comparison with future imprinted polymers⁹. During the batch rebinding experiment, metergoline was allowed to bind with MIP and NIP until equilibrium was reached. At this point the rate of binding and unbinding is equal and a steady state will set in between the concentration of free analyte in solution and the quantity bound to the polymer. This equilibrium approach allows omitting the time dependency. The binding experiments for MIP and NIP were executed two times; first with 50 mg of polymer which is in accordance with the application in the SPE column and secondly with 5 mg of polymer. It can be expected that the former will be capable of binding higher amounts of compound from the solution and thus show an enhanced dynamic range towards higher concentrations. However, literature suggests that binding efficiency increases with respect to the quantity of compound bound per unit of mass of MIP with decreasing amount of MIP used^{32,31}. Therefore, also 5 mg of polymer was tested in order to estimate the influence of this mass dependency.

It is apparent from the binding isotherms (Figure 3) that the imprinted polymer binds higher amounts of metergoline than its non-imprinted counterpart for both amounts of sorbent tested. The amount of compound bound to the NIP can be attributed entirely to non-specific binding. The additional bound analyte to the MIP in comparison with the NIP can be ascribed to binding of metergoline to binding sites with higher specificity. The graph also shows that the binding will be more efficient when low concentrations of analyte are applied. This is an interesting observation if one keeps in mind the use of the MIPs in the clean-up procedure. Often, samples

contain only trace amounts of ergot alkaloids and in these cases it is important to have a sensitive clean-up where loss of analytes is limited in order to reduce false negative results.

As anticipated, the amount of metergoline bound to the MIPs increased when a smaller quantity of polymer was used. However, a severe loss in recovery of the ergot alkaloids was established during the MISPE procedure with cartridges packed with 5 mg of MIP compared to the same procedure executed with 50 mg packed cartridges. This is probably due to the low amount of polymer which provides insufficient back-pressure and thus impedes sufficient interaction time. Therefore, the columns were packed with 50 mg of polymer which resulted in a good balance between the recovery criterion and an easy manageable column. However, the enhanced performance of the MIP at lower amounts is important in for example sensor applications where even minute changes in mass can be measured.

Figure 3. Binding isotherms obtained during batch rebinding experiments. The upper two curves represent the MIP and NIP respectively, using 5 mg of polymer during rebinding. The lower two curves and their magnification in the right box are the isotherms obtained using 50 mg of polymer.

Scatchard analysis If the polymer has more than one group of binding sites, a curved line is obtained in the Scatchard plot rather than a straight line³⁵. In the case of one ligand (metergoline) and two types of binding sites (one type of site with high and one with low selectivity) two straight lines would be generated with different slopes according to the Scatchard equation. However, the experimentally obtained line was again curved. Using the non-covalent approach, the carboxylic groups of the functional monomer can interact with up to three amine groups present on the template to form the pre-polymerization complex. Due to incomplete association during this complexation, a variety of binding spots with different selectivity occurred in the MIP.

Occupation of the binding sites with the highest affinity was predominant in the low concentration range and binding with the lowest affinity sites occurred in the high concentration range. All the other binding sites showing a variety of binding affinities between these two extremes, were located in the intermediate area. Therefore, the values of B_{max} and K_D should be considered as *apparent* values. They can be used for comparative purpose but cannot be regarded as absolute values considering the assumptions made in the Scatchard approach^{32,31,35}. However, they are a good indicator for the presence of different binding sites on the MIP as a curved experimental slope is observed. Especially when compared to the NIP which can easily be represented by a straight line. The observation of the different slope behavior for MIP and NIP indicates different binding behavior.

Application of equation 1 to the values of the binding isotherms renders the plot and the corresponding values for the apparent B_{max} and K_D as displayed in Figure 4. The NIP curve shows a linear slope indicative of one type of binding sites possessing a low selectivity. The MIP on the other hand displays different slopes, which indicates the presence of heterogeneous binding sites. Two slopes are distinguished, one which represents the binding sites with higher affinities (MIP high) and another with lower affinity (MIP low). The high-selective binding spots have a lower dissociation rate, and are present in smaller amount than the low-selective sites. The overall affinity of the MIP towards the target compound is higher than compared to the NIP, as displayed by the K_D values.

Figure 4. Scatchard plot of the MIP and NIP isotherm. The ratio bound amount to free amount of metergoline in solution (ml/g) is plotted to the amount of metergoline bound to the MIP or NIP (nmol/mg).

Matrix effect

Obviously, a good recovery and the removal of matrix interferences are related to the use of a proper washing solvent. If the analyte shows a high affinity towards the washing solvent compared to the polymer matrix, the analyte will be washed off and the recoveries will be low. On the other hand, a weak washing solvent may not remove matrix interferences and other contaminants sufficiently.

Organic solvents such as methanol, acetonitrile, acetone and hexane were used during the washing step in order to remove neutral or hydrophobic compounds such as for example non-polar lipids³³. However, they all led to a considerable loss in recovery. After extraction, a quantity of water-soluble compounds such as pectin, monosaccharides, proteins, amino acids, etc... is expected to be found in the sample^{33,34}. This is because the extraction solvent comprises a part of NH_4HCO_3 buffer to obtain the desired pH. Therefore, combinations of the previously mentioned organic solvents with different aqueous solvents in different ratios were also tested but led to a severe loss of recovery as well. This can be explained by the fact that the ergot alkaloids are soluble in organic solvents, especially the protic organic solvents such as methanol. The ionic interactions between the polymer and the ergot alkaloids were not sufficiently strong to compete with the solubilizing capacity of the solvents used in these experiments. Data of these tests are not included in this paper.

When washing with water, good recovery values were obtained as could be expected considering the rather hydrophobic nature of the ergot alkaloids (Table 2). A value of 100% indicates no matrix effect whereas lower values indicate ion suppression. All compounds were prone to ion suppression but most of them experienced limited influence due to the matrix. However, ergometrine and ergometrinine eluted in an early stage together with the compounds that possessed low affinity towards the column and were heavily affected by these latter. Figure 5 illustrates post-column injection experiments, conducted to compare the MISPE clean-up to the procedure used by Diana Di Mavungu et al¹⁰. Again, the strong influence of the matrix was observed at the beginning of the analysis for both methods. A similar behavior for ergotamine was observed during the continuation of the analysis with the MISPE-prepared samples being slightly less prone to ion suppression. This indicates that the MISPE clean-up procedure performed equal to the regular preparation in terms of matrix effect. The same profiles were obtained for the other ergot alkaloids, but are not included in this article.

Finally, aprotic solvents such as ethyl acetate, diethyl ether and DMF were used to attempt the removal of hydrophobic interferences. Aqueous solutions of salts such as NaCl and MgSO_4 were applied to precipitate proteins. Although some mixtures presented the same recoveries compared to the use of water as washing solvent, no improvements were observed regarding the matrix effect.

In conclusion, 2 ml of distilled water was used as washing solvent as mentioned in the experimental part. The choice for this solvent was based on the overall good performance on two criteria: recovery of the analyte and matrix effect.

Em	Emn	Es	Et	Eco	Ekr	Ecr	Esn	Etn	Econ	Ekrn	Ecrn
5 ± 1	11 ± 3	89 ± 12	96 ± 12	83 ± 15	80 ± 13	69 ± 9	82 ± 15	68 ± 11	91 ± 12	92 ± 14	92 ± 11
6 ± 3	14 ± 5	90 ± 17	94 ± 20	78 ± 18	78 ± 19	69 ± 19	94 ± 7	79 ± 9	102 ± 18	107 ± 13	109 ± 14

Table 2. Matrix effect (in %) and standard deviation (in %) of barley samples with water as washing solvent (n = 4). First row: matrix effect for the MIP; second row: matrix effect for the NIP.

Figure 5. Post-column injection to evaluate the effect of barley matrix on ergotamine. Blue: standard ergotamine solution, no matrix. Green: injection of a sample prepared according to the clean-up of Diana Di Mavungu et al¹⁰. Red: sample after MISPE clean-up.

Cross-reactivity

The cross-reacting mycotoxins could be classified into different groups according to their affinity towards the MIP sorbent. This difference was expressed as recovery (in %) in Figure 6 and could be related to the various conformations.

The first group displayed recoveries below 13% meaning there was low to no cross-reactivity. Some of the compounds comprised in this group such as NIV, DON, NEO and F-X, are hydrophilic. During the washing step with water, they were easily removed. If one considers the partition coefficients, log P, and the water solubilities of the fumonisins (FB-1, FB-2, FB-3), one would not predict their low affinity towards the SPE sorbent. However, due to the presence of carboxylic acid functionalities and the corresponding low pKa values, these compounds were not able to form a cation during the loading step (pH = 7) and were therefore unable to interact with the particles. This also applied to CIT and OTA.

A high level of cross-reactivity was shown in the second group with recovery values greater than 40%. As could be predicted by their log P values and/or low solubility in water, these compounds showed little affinity towards water. The pKa of these substances is situated in the basic area, indicating no anions will be formed but rather neutral or positively charged molecules. Contrary to the first mentioned group, this led to enhanced interaction with the MIP polymer matrix. BEAU and Roq.C also possess tertiary amine functional groups which can engage non-specific interactions with the MAA's acidic functional groups, thus adding to the affinity towards the sorbent.

An intermediate cross-reactivity was displayed in the third group with recoveries between 20% and 40%. All of the compounds in this set are poly-cyclic structures which, on average, have lower log P values than the ones in group 2, indicating a tendency to dissolve better in water. However, none of the compounds hold a carboxylic acid which impedes the fluent elution and low recoveries as seen in group 1.

There was no statistical difference (using t-tests and p-values, not included in this article) between the recoveries for MIP and NIP towards the different mycotoxins, not even for tertiary amine containing substances such as Roq.C. This is an important result because it indicates that the cross-reactivity of these 25 mycotoxins can be attributed to non-specific interactions. This is in clear contrast with the recoveries for the ergot alkaloids (+ epimers) which showed a clear difference between MIP and NIP, related/derived from the specific interactions of the analyte with the MIP cavities. The use of both imprinted and non-imprinted polymer allowed distinguishing between the selective and non-selective binding.

Figure 6. Cross-reactivity study performed on barley samples in triplicate. The recovery values (in %) for the 25 mycotoxins towards MIP (red) and NIP (black) are plotted.

Conclusions

Molecularly imprinted polymers were successfully developed towards twelve ergot alkaloids: ergometrine, ergotamine, ergocornine, ergosine, ergocryptine, ergocristine as well as their respective epimers. Metergoline was used as a template molecule for the first time together with methacrylic acid and ethylene glycol dimethacrylate monomers in suspension polymerization. This approach produced spherical beads with a narrow size distribution which were applied as a sorbent in an SPE column and used for the clean-up of barley samples prior to LC-MS/MS analysis.

It was the first time a group-selective MIP was developed against these ergot alkaloids and the use of NIP allowed discrimination of non-selective binding events. The imprinted polymer performed well in comparison with traditional clean-up with good recoveries, reduced matrix effect for most compounds and low detection limits. It offers advantages by its easy and cheap production, short application time of the MISPE procedure, reduced use of consumables, milliliter-amount necessity for solvents and its reusability.

The beneficial influence of using the suspension polymerization method instead of bulk polymerization was demonstrated. It was also pointed out that the use of different amounts of polymer had an effect on the binding efficiency. However, this holds a potential use of these MIPs in other applications as well, for example as sensitive layer in sensor applications.

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